REMARKS

Claims 1-4, 7, 9-10, 20-22, 25-26, 33-35, 38-39, 46-47 and 50-51 were pending. Applicant currently amends Claims 1, 20 and 46 and cancels Claims 7, 22, 33-35, 38-39 and 47 herein without prejudice. Thus, Claims 1-4, 9-10, 20-21, 25-26, 46 and 50-51 are currently pending.

The amendments to independent Claims 1, 20 and 46 merely add the limitations of dependent Claims 7, 22 and 47, respectively. The amendments do not involve new matter and support for the amendments can be found as follows.

Support for amended Claim 1 can be found in originally filed Claim 7.

Support for amended Claim 20 can be found in originally filed Claim 22.

Support for amended Claim 46 can be found in originally filed Claim 47.

Accordingly, these amendments to the claims do not involve new matter and their entry is respectfully requested.

THE CLAIMED INVENTION

Before Applicants' invention, no one taught or suggested using sulfhydryl protected glutathione prodrugs to replenish GSH in a subject. Applicants were the first to use sulfhydryl protected glutathione prodrugs as a readily accessible source of GSH. Applicants were the first to provide experimental evidence that an exogenously administered sulfhydryl protected glutathione prodrug (e.g., L-CySSG) can protect the liver from the toxic insult of acetaminophen, a drug known to severely deplete GSH and elicit hepatotoxicity.

Advantage of the invention: A sulfhydryl protected glutathione prodrug releases a preformed glutathione making it immediately available to the cell. Release of GSH from a sulfhydryl

protected glutathione prodrug bypasses the cellular GSH synthesis pathway providing GSH independent of whether the pathway is functional or not. Additionally, the sulfhydryl protected glutathione prodrug protects GSH from degradation by the cell.

For example, in an embodiment of the invention, the sulfhydryl protected glutathione prodrug is CySSG (also known as CSSG), where CySSG is a mixed disulfide of L-cysteine and glutathione. When reduced, CySSG releases a preformed glutathione as well as L-cysteine. The L-cysteine is a precursor for *de novo* glutathione synthesis. Thus, CySSG produces two glutathiones; the first glutathione is released from CySSG by reduction making it immediately available to the cell; the second glutathione is *de novo* synthesized by the cell from the L-cysteine that was released by CySSG (specification at page 9, first paragraph).

REJECTION UNDER 35 U.S.C. §103(a)

The Examiner rejected claims 1-4, 7, 9-10, 20-22, 25-26, 33-35, 38-39, 46-47 and 50-51 as unpatentable over Shirota et al., Jonas et al. and Bender et al. **The Office took the following positions.**

1. The combined prior art references suggested the present invention because:
    a. it would have been obvious to substitute CySSG (Jonas et al.) for CySSME (Shirota et al.) for the production of GSH through cysteine production (April 11, 2007, Office Action, page 4, paragraph 2 and page 11, last paragraph); and
    b. one skilled in the art would recognize the nexus between cysteine production and GSH levels, important for regulating oxidative stress (April 11, 2007, Office Action, page 3, paragraph 3, page 6, last paragraph to page 7, line 2 and page 11, paragraph 2).
2. One skilled in the art would have been motivated to administer CySSG to induce intracellular Cystine (as described in Jonas et al.), whereupon the cell converts cystine to cysteine, the rate limiting precursor of GSH, and the reduction of oxidative stress (Bender et al.; April 11, 2007 Office Action, page 3, paragraph 3).

3. One of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention (i.e., a method for reducing oxidative stress using a sulfhydryl protected glutathione prodrug) because CSSG has already been administered to cells and cysteine levels were increased three fold (April 11, 2007 Office Action, page 3, paragraph 3 and page 10, paragraph 2).
4. CySSG is NOT a sulfhydryl protected glutathione prodrug (as asserted by Applicants) but rather a cysteine prodrug (April 11, 2007, Office Action, page 4, last line to page 5, line 3, page 8, paragraph 2 and page 10, paragraph 2).

Applicants respectfully disagree. However, in order to further prosecution of the subject application, Applicants have cancelled Claims 33-35, 38 and 39. With respect to remaining Claims 1-4, 7, 9-10, 20-22, 25-26, 46-47 and 50-51, Applicants maintain their traversal as follows.

ARGUMENT

Teachings of the Cited Art

Shirota et al. teach that L-cysteine prodrugs such as CySSME (a mixed disulfide of cysteine and mercaptoethanol), can reduce acetaminophen induced hepatic toxicity due to enhanced GSH synthesis and maintenance of hepatic GSH homeostasis. CySSME is not a sulfhydryl protected glutathione prodrug. Shirota does not teach the use of a sulfhydryl protected glutathione prodrug.

Jonas et al. teach a use of a sulfhydryl protected glutathione prodrug, i.e. CSSG, to provide "a soluble source of cyst(e)ine" for cystinotic cells, cells from patients heterozygous for cystinosis and normal (i.e., non-cystinotic) cells to increase cystine levels in the cells. Jonas did not teach or suggest use of CSSG to increase GSH levels in a cell.

Bender teaches that in cultured astrocytes cellular uptake of cystine is the rate-limiting step in GSH biosynthesis. Cystine, after transport into cells, is reduced to cysteine, a precursor of GSH. Thus, cystine, via cysteine, is required for maintaining cellular levels of GSH. GSH protects cells against oxidative stress and various toxins. Bender does not teach or suggest increasing GSH levels using cystine or any other molecule, let alone a sulfhydryl protected glutathione prodrug.

The Prior Art References Did Not Suggest the Invention

The Office alleges that the combination of cited references suggested the claimed invention since Bender's teaching (cystine is reduced to cysteine which is used to *de novo* synthesize glutathione) provides a nexus for substituting CSSG (Jonas et al.) in place of a L-cysteine prodrug such as CySSME (Shirota et al.) for the production of GSH thereby reducing oxidative stress (April 11, 2007 Office Action, page 3, paragraph 3, page 6, last paragraph to page 7, line 2 and page 11, paragraph 2).

This reasoning falsely assumes that:
(1) there is similarity between CSSG and CySSME which would suggest their interchangeability;
(2) there is motivation to use sulfhydryl protected glutathione prodrug instead of a cysteine prodrug; and
(3) motivation exists to use sulfhydryl protected glutathione prodrug to reduce oxidative stress.

There is no similarity between CSSG and CySSME. CSSG is a sulfhydryl protected *glutathione* prodrug. In contrast, CySSME is a sulfhydryl protected *cysteine* prodrug (specification at page 3, lines 9-12). CySSME is a mixed disulfide of L-cysteine and mercaptoethanol. Only a single glutathione can be produced from CySSME, this via *de novo* synthesis.

There is no motivation to substitute CSSG for CySSME for reducing oxidative stress in a cell. CSSG and CySSME do not share a common utility and function (In re Lalu and Foulletier 747 F.2D 703, 223 USPQ 1257 (Fed. Cir. 1984)). They are not equivalents.

As the Office states, Shirota teaches that hepatoprotection by L-cysteine generated from a prodrug is due to enhanced GSH **synthesis** (April 11, 2007, Office Action, page 10, paragraph 3). Neither Jonas nor Bender teach or suggest what Shirota fails to teach or suggest, namely, the use of sulfhydryl protected glutathione prodrug to deliver **preformed** glutathione to cells or to reduce oxidative stress in a cell. Accordingly, the combination of the primary and secondary references does not and cannot render obvious the claimed methods.

The mere fact that references can be combined does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 U.S.P.Q.2d 1430, cited in MPEP §2143.01. There must be a reason or suggestion in the art for modifying the prior art other than the knowledge learned from Applicants' disclosure[1]. However, the cited references provide none.

The Benefits of Using a Sulfhydryl Protected <u>Glutathione</u> Prodrug

Unlike sulfhydryl protected *<u>cysteine</u>* prodrugs, administration of a sulfhydryl protected *<u>glutathione</u>* prodrug to a cell provides a source of *<u>preformed glutathione</u>* independent of the cell's endogenous GSH biosynthesis pathway. As an organism ages, the GSH synthesis pathway becomes impaired leading to a decrease in GSH biosynthesis. Sulfhydryl protected glutathione prodrugs such as CSSG deliver preformed GSH to the cell whether or not the GSH biosynthesis pathway is functional.

Additionally, to alleviate aggressive oxidative stress, for example after acetaminophen administration, a source of GSH such as a sulfhydryl protected glutathione prodrug can be provided expeditiously to a cell. Prior to the present invention, it was not obvious that sulfhydryl protected glutathione prodrugs were a readily available and efficient source of GSH to a cell nor to administer a sulfhydryl protected glutathione prodrug such as CSSG since, among other

[1] *In re Dow Chemical Co.*, 837 F.2d 469, 473, 5 USPQ2d 1529, 1532 (Fed. Cir. 1988).

things, it had not been known whether the enzymatic reduction (or enzyme-catalyzed thiol-disulfide interchange reaction) velocity of CSSG would be sufficiently fast and efficient to replenish GSH.

There Was No Reasonable Expectation of Success for the Substitution of a Sulfhydryl Protected Glutathione Prodrug for a Cysteine Prodrug

In response to the Applicants statement that there was no reasonable expectation of success that the substitution of a sulfhydryl protected glutathione prodrug for a cysteine prodrug, sufhydryl-protected or otherwise, would produce the claimed methods, the Office stated that "[b]oth CySSME and CYSSG (CSSH) (sic) were shown to produce an increase in cysteine levels which are important for producing GSH. Therefore, it worked and was successful" (April 11, 2007, Office Action, Page 10, paragraph 2).

Respectfully, the Office misses the point. Although GSH can be generated from cysteine prodrugs by de novo synthesis in a cell, whether cysteine is converted into GSH depends on several factors: the type of cell, the amount of cysteine present, the amount of GSH present, etc. For example, if a cell has sufficient GSH, the GSH synthesis pathway may be turned off. Alternatively, the GSH pathway may be blocked by an inhibitor or impaired. Whether a molecule can increase cysteine is not pertinent to the claimed invention.

Moreover, not all compounds containing cysteine (e.g., cysteine prodrugs) are substitutable for CySSME to increase cysteine levels. Some cysteine prodrugs may increase cysteine levels but others do not (Crankshaw et al., J Biochem Mol Tox, 2002, 16(5):235-244 (Exhibit 1).

Even if, *arguendo*, CSSG could be substituted for CySSME to increase cysteine levels, the claims are not directed to increasing cysteine levels. *The claims are directed to uses of sulfhydryl protected glutathione prodrugs to reduce oxidative stress, increase GSH in a cell or reduce hepatotoxicity.* In the claimed methods, the GSH can be rapidly released from the prodrug and

provided to the cell even if the endogenous cellular GSH biosynthesis pathway is broken. The sulfhydryl protected glutathione prodrug protects GSH from degradation by the cell.

Jonas administered CSSG to increase intracellular cysteine, but did not disclose that CSSG administration would provide preformed GSH to reduce oxidative stress in a subject as claimed. The Shirota and Bender references do not supply what Jonas lacks. Given the state of the art, there would have been no reasonable expectation that one would be able to produce the claimed method by simply administering to a subject a sulfhydryl protected glutathione prodrug such as CSSG.

Although obviousness under 35 U.S.C. §103 does not require absolute predictability of success, 35 U.S.C. §103 does require a reasonable expectation of success to find obviousness. (In re O'Farrell, 853 F.2d 894, 7 USPQ2d 1673 (Fed. Cir. 1988)).

The References Were Not Found in the Applicants Disclosure but Through a Literature Search

Applicants wish to correct a misunderstanding. The Office appears to have misunderstood Applicants' statement in the response to the Office Action dated November 15, 2006. Applicants stated that the teaching or suggestion to make the claimed combination, and the reasonable expectation of success, must both be found in the prior art, not in Applicants' disclosure. This was not meant to suggest that the art cited by the Office was found in the application.

CySSG Is a Sulfhydrydral Protected Glutathione Prodrug

The Office's statement that CySSG is NOT a sulfhydryl protected glutathione prodrug is contrary to the definition of the generally accepted term "prodrug" as well as contrary to the specification at page 3, lines 9-14 (for support see attached definition of prodrug from the Oxford Dictionary of Biochemistry and Molecular Biology (2003), Oxford University Press Inc. (Exhibit 2)). MPEP 2111.01 provides that the Office must give the words of the claim their plain meaning. This is especially important when applicants provide a definition of a claim term in accord with its accepted meaning.

CONCLUSION

If a telephone interview would be of assistance in advancing the prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone her at the number provided below.

No fee is deemed necessary in connection with the filing of this Communication. If any fee is necessary, the Patent Office is authorized to charge any additional fee to Deposit Account No. 50-0306.

Respectfully submitted,

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EXHIBIT 1

number of other dyes useful in biochemistry, especially in **dye-ligand chromatography**.

procoagulant *(sometimes)* any of factor V, factor VII, or factor VIII, each of which acts to accelerate the conversion of prothrombin to thrombin during **blood coagulation**.

procollagen a ≈150 kDa triple-helical protein that is an intermediate in the biosynthesis of **tropocollagen**, into which it is converted proteolytically by removal of the additional sequences at both the N and the C termini of each of the three peptide chains. Example, procollagen α1 (human): database code CA11_HUMAN, 1464 amino acids (138.73 kDa). *Compare* **protocollagen**.

procollagen *N*-endopeptidase EC 3.4.24.14; *other name*: procollagen *N*-proteinase; an enzyme that catalyses the cleavage of the *N*-propeptide of collagen chain α-1(I) at Pro-|-Gln and of chain α-2(II) at Ala-|-Gln.

procollagen-lysine 5-dioxygenase EC 1.14.11.4; *other names*: procollagen-lysine,2-oxoglutarate 5-dioxygenase; lysine hydroxylase; lysine,2-oxoglutarate 5-dioxygenase; an enzyme that catalyses the formation of hydroxylysine residues in collagen. The reaction is between dioxygen, procollagen L-lysine, and 2-oxoglutarate to form procollagen 5-hydroxy-L-lysine, succinate, and CO_2. Iron and **ascorbic acid** are cofactors. Example (precursor) from human: database code LYSH_HUMAN, 727 amino acids (83.49 kDa). *See also* **procollagen-proline dioxygenase**.

procollagen-proline dioxygenase EC 1.14.11.2; *systematic name*: procollagen-L-proline,2-oxoglutarate:oxygen oxidoreductase (4-hydroxylating); *other names*: proline,2-oxoglutarate-4-dioxygenase; protocollagen hydroxylase; proline hydroxylase; prolyl 4-hydroxylase. An enzyme converting L-proline into 4-hydroxy-L-proline; it catalyses a reaction between dioxygen, procollagen L-proline, and 2-oxoglutarate to form procollagen *trans*-4-hydroxy-L-proline, succinate and CO_2. Hydroxylation only occurs when proline is present in a polypeptide chain elongating on a ribosome. **Ascorbic acid** and Fe^{3+} are also required as cofactors, and the reaction is one of the few well-defined roles for ascorbic acid; it seems likely that scurvy, as a result of vitamin C deficiency, is due to defective activity of this enzyme, which plays a key role in collagen synthesis. The enzyme is a tetramer of two α chains and two β chains; the β chain is the multifunctional **protein disulfide isomerase** and hence has microsomal triacylglycerol transfer protein activity. Subcellular location is the endoplasmic reticulum lumen. There are two forms of α subunit, which are produced by alternative splicing of the same gene. Example from human (α precursor): database code P4HA_HUMAN, 534 amino acids (60.84 kDa). See also **procollagen-lysine 5-dioxygenase**.

proconvertin *an alternative name for* factor VII; *see* **blood coagulation**.

procorticotropin *or* **procortin** *an alternative name for* **proopiomelanocortin**.

procyclin *or* **PARP** a major surface antigen of procyclic forms of trypanosomes. It is GPI-anchored. Example B1-α precursor from *Trypanosoma brucei brucei*: database code PAR1_TRYBB, 143 amino acids (14.88 kDa).

prodrug a drug molecule that is itself inert but has pharmacological effects after **bioactivation**. An example is **ganciclovir**.

produce *(in mathematics)* to extend a line or a plane.

product 1 *(in biochemistry and chemistry)* something formed in a reaction. 2 *(in mathematics)* the result of multiplying together two or more numbers, quantities, or expressions.

product inhibition the inhibition of an enzymic reaction caused by increased concentration of one or more products of that reaction.

productive (capable of) yielding a product, result, or benefit.

productive binding *or* **eutopic binding** the binding of a substrate in a reactive mode at the active site of an enzyme. *Compare* **nonproductive binding**.

productive complex any enzyme–substrate complex in which the substrate is bound to the enzyme in a manner that renders catalysis possible so that products can be formed. *Compare* **abortive complex**.

productivity *(in biotechnology) symbol*: r; the mass of product formed per unit reactor volume per unit time; often per unit of enzyme or biomass. It is measured typically in kg m^{-3} h^{-1}.

proenkephalin a protein precursor for several neuropeptides including various **enkephalins** formed in brain and adrenal medulla. For example, in the human, proenkephalin A precursor yields four copies of [Met]enkephalin (100–104; 107–111; 136–140, 210–214) and single copies of each of [Leu]enkephalin (230–234), [Met]enkephalin–Arg–Gly–Leu (octapeptide) (186–193) and [Met]enkephalin–Arg–Phe (heptapeptide) (261–267), which arise by enzymatic cleavage of the gene product by cleavage at paired basic amino acids: database code PENK_HUMAN, 267 amino acids (30.79 kDa). Another human protein, proenkephalin B precursor (*other name*: β-neoendorphin–dynorphin precursor) yields single copies of α-neoendorphin, dynorphin, [Leu]enkephalin, rimorphin, and leumorphin, also by cleavage at paired basic amino acids: database code NDDB_HUMAN, 254 amino acids (28.38 kDa).

proenzyme *an alternative name for* **zymogen**.

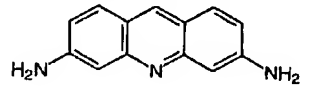
***pro-E*/*pro-Z* convention** if the two chemically-like hydrogen atoms of $abC{=}CH_2$ are separately replaced by the achiral, *d*, an achirotopic stereogenic element is generated and two (diastereoisomeric) alkenes, $abC{=}CHd$ are formed that are *E* and *Z* stereoisomers (*see* ***E*/*Z* convention**). The replacement has converted a prostereogenic element into which one which is stereogenic; the situation is comparable to the change, prochiral → chiral, although a chiral element is not involved. The two chemically-like hydrogen atoms are designated as *pro-E* and *pro-Z*, and are abbreviated as H_E and H_Z. Replacement of a *pro-E* hydrogen with ^2H yields an *E* diastereoisomer and the similar replacement of a *pro-Z* hydrogen yields the *Z* form. Example: the two hydrogen atoms of phospho*enol*pyruvate; that on the same side of the double bond as the COOH group is H_E, that on the same side as the OPO_3H_2 group is H_Z. Replacement of ^1H_E by ^2H yields (*E*)-[3-$^2H_1$]phospho*enol*pyruvate; the *Z* diastereoisomer results if ^1H_Z is replaced by ^2H. A similar situation is found in the side chain of **chorismic acid**.

proestrus *see* **estrous cycle**.

profibrinolysin *an alternative name for* **plasminogen**.

profilin a protein that *in vitro* prevents the polymerization of actin. In many cells it occurs as a 1:1 complex with monomeric actin, that appears to act as an intracellular storage form of actin. Profilin binds $PtdIns{P_2}$ and inhibits the γ isoform of phospholipase C (*see* **phospholipase**), an inhibition that is overcome when the enzyme is activated as a result of receptor kinase-dependent tyrosine phosphorylation. This activation appears in some way to recruit profilin–actin to the site of filament assembly which occurs when cells are stimulated e.g. by nitrogen. Example, profilin I (human): database code PRO1_HUMAN, 139 amino acids (14.91 kDa).

proflavin 3,6-diaminoacridine; an **acridine** dye, molecules of which are capable of intercalating between adjacent base pairs in duplex DNA. It is useful as an antibacterial agent, by virtue of its ability to inhibit the biosynthesis of both DNA and RNA, possibly through intercalation into preexisting DNA, and as a frameshift mutagen for bacteriophages.



progesterone *the common name for* pregn-4-ene-3,20-dione; the principal steroid hormone of the corpus luteum, from which it is secreted during the latter half of the estrous cycle, acting upon the endometrium to prepare it for embryo im-

Oxford Dictionary of Biochemistry & Molecular Biology, 2003
Oxford University Press.

EXHIBIT 2

J BIOCHEM MOLECULAR TOXICOLOGY
Volume 16, Number 5, 2002

Double-Prodrugs of L-Cysteine: Differential Protection Against Acetaminophen-Induced Hepatotoxicity in Mice

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ABSTRACT: A series of double-prodrugs of L-cysteine, designed to release L-cysteine in vivo and stimulate the biosynthesis of glutathione (GSH), were synthesized. To evaluate the hepatoprotective effectiveness of these double-prodrugs, male Swiss-Webster mice were administered acetaminophen (ACP) (2.45 mmol/kg (360 mg/kg), intraperitoneally (i.p.)). Prodrug (2.50 mmol/kg, i.p. or 1.25 mmol/kg, i.p., depending on the protocol) was administered 1 h before ACP as a priming dose. A supplementary dose of prodrug (2.5 mmol/kg, i.p. or 1.25 mmol/kg, i.p. depending on the protocol) was administered 0.5 h after ACP. The plasma alanine amino transferase (ALT) values, 24 h after ACP administration were transformed to logs and the 95% and 99% confidence intervals of the log values were plotted and compared for each group. Hepatoprotection was assessed by the degree of attenuation of plasma ALT levels. With these multiple dose schedules, the use of 2% carboxymethylcellulose as vehicle for the prodrugs was found to be detrimental; therefore, the prodrugs were dissolved in dilute aqueous base and the pH adjusted for administration. When a priming dose was given 1 h before ACP followed by a supplementary dose 0.5 h after ACP, only *N,S*-bis-acetyl-L-cysteine, where both the sulfhydryl and amino groups of L-cysteine were functionalized with the acetyl group, was found to be effective in protecting mice against the hepatotoxic effects of ACP. This suggests that these acetyl groups were rapidly hydrolyzed in vivo to liberate L-cysteine. In contrast, *N*-acetylation of 2(*R*,5)-methylthiazolidine-4(*R*)-carboxylic acid (MTCA) and its 2-*n*-propyl analog (PTCA), or *N*-acetylation of 2-oxothiazolidine-4-carboxylic acid (OTCA), reduced the hepatoprotective effects relative to the parent MTCA, PTCA, and OTCA, indicating that the release of L-cysteine in vivo from these *N*-acetylated *thiazolidine* prodrugs was metabolically unfavorable. The carbethoxy group, whether functionalized on the sulfhydryl or on the amino group of L-cysteine, or on the secondary amino group of MTCA, appears to be a poor "pro-moiety," since these carbethoxylated double-prodrugs of L-cysteine did not protect mice from ACP-induced hepatotoxicity. © 2002 Wiley Periodicals, Inc. J Biochem Mol Toxicol 16:235–244, 2002; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.10044

KEYWORDS: L-Cysteine; Hepatotoxicity; Acetaminophen; Glutathione; Prodrugs

INTRODUCTION

L-Cysteine is the rate-limiting sulfhydryl amino acid required for the first step in the two-step biosynthesis of glutathione (GSH) [1]. Prodrugs of cysteine are, therefore, also GSH precursors, since the cysteine liberated in vivo from their prodrug forms stimulates GSH biosynthesis and is incorporated into this tripeptide. It is now well established that L-cysteine prodrugs [2–7], or prodrugs of GSH itself [8,9], are effective hepatoprotective agents that can greatly attenuate the liver toxicity elicited in rodents by high doses of toxic xenobiotics such as acetaminophen (ACP). The hepatotoxicity of ACP is generally believed to be a consequence of the formation of a highly reactive metabolic oxidation product of ACP, viz., *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which binds ubiquitously to tissue macromolecules, thereby triggering a cascade of inflammatory events eventually leading to necrosis [10]. GSH effectively sequesters this reactive metabolite of ACP and protects the liver from toxicity [11].

L-Thiazolidine-4-carboxylic acid (TCA) and its 2-oxo- (OTCA) and 2-alkyl-substituted (MTCA, PTCA) derivatives (Chart 1) serve as prodrugs of L-cysteine. Bioactivation of TCA requires the intervention of the hepatic mitochondrial enzyme, proline oxidase [5,12,13], to give Δ^2-thiazoline-4-carboxylic acid, which

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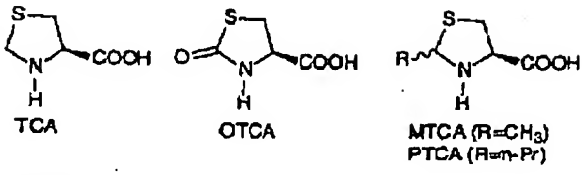


CHART 1.

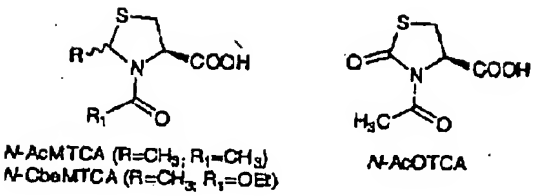
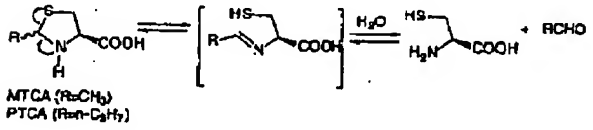


CHART 2.

spontaneously hydrolyzes nonenzymatically to *N*-formyl-L-cysteine (structures not shown). The latter is further hydrolyzed by a cytosolic enzyme to formate and L-cysteine as TCA can replace L-cysteine as a nutritional component in rats [14]. Similarly, the enzyme 5-oxoprolinase is required to open the thiazolidine ring of OTCA to give L-cysteine, this process requiring 1 mol of ATP [15].

In contrast, MTCA and PTCA are hydrolytically labile, and, under physiological conditions of temperature and pH, undergo a nonenzymatic, hydrolytic ring opening dissociation to liberate L-cysteine and acetaldehyde or propionaldehyde [4,5] (see Eq. (1)). A "demand-pull" dissociation is operational in vivo when either the aldehyde or cysteine is removed by metabolic action. In vitro, the presence of L-cysteine in the medium suppresses this dissociation [5], while volatilization of the aldehyde (RCHO) or removal of the cysteine, e.g., by air oxidation, promotes dissociation. Indeed, the possibility of hydrolytic dissociation of MTCA and PTCA mandates that, for chemical or biological



(1)

studies, H_2O solutions of these compounds be prepared fresh, just prior to use. It follows that, unlike TCA or OTCA, aqueous diet preparations containing these prodrugs cannot be preformulated, placing severe restrictions on any animal feeding experiments that may be contemplated with MTCA and/or PTCA.

However, by acylating the secondary amino (N—H) function of the thiazolidine, e.g., with an acetyl (Ac) or a carbethoxy (Cbe) group, MTCA is converted to stable derivatives (Chart 2) that cannot hydrolyze spontaneously and now require *enzymatic* deacylation of the *N*-acetyl or the *N*-carbethoxy group to convert them back to MTCA. Similarly, enzymatic deacetylation of the *N*-acetylated OTCA (*N*-AcOTCA, Chart 2) would result in reversion of this compound to OTCA.

These *N*-acylated thiazolidine-4-carboxylic acids can, therefore, be considered to be *double*-prodrugs of L-cysteine, inasmuch as two sequential steps (enzymatic/nonenzymatic or double enzymatic) are required for the liberation of this sulfhydryl amino acid in vivo.

N-Acetyl-L-cysteine (*N*-AcC, Chart 3), a well-known cysteine prodrug (2), provides the free sulfhydryl amino acid to cells following its rapid enzymatic deacetylation in the liver [16]. *N*-AcC is the only FDA-approved antidote for the treatment of hepatotoxic drug overdoses, such as with ACP, a widely used (and misused) analgesic agent. While effective clinically, *N*-AcC has an unpleasant taste and is known to have poor bioavailability (9% in humans, hence large doses are required) [17] and is extensively covalently bound to plasma proteins (>50%) because of the presence of the free, reactive sulfhydryl group [10]. Except for the thiazolidine-4-carboxylic acids, where this SH group of L-cysteine is latentiated, i.e., chemically protected until uncovered either by metabolic action or by nonenzymatic hydrolysis [5–7], sulfhydryl-protected L-cysteine derivatives have not been studied as protective agents against ACP-induced hepatotoxicity [10]. Accordingly, *N,S*-di-acylated derivatives of L-cysteine were synthesized (Chart 3) as a second series of sulfhydryl-protected, double-prodrugs for comparison with the thiazolidine double-prodrugs listed in Chart 2. *N*-Carbethoxy-L-cysteine (*N*-CbeC), although itself not a *double-prodrug*, was also prepared for comparison

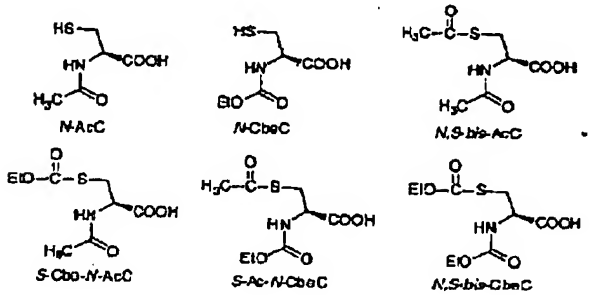


CHART 3.

TABLE 1. Prodrug/ACP Administration Protocols Using Various Vehicles

	Time (min)		
Protocol (Vehicle)	*−60*	*0*	*+30*
1 (CMC)	Prodrug (2.50)	ACP (2.45)	Prodrug (1.25)
2 (CMC)	Prodrug (2.50)	ACP (2.45)	–
3 (pH adjusted saline)	Prodrug (2.50)	ACP (2.45)	Prodrug (1.25)
4 (pH adjusted saline)	Prodrug (1.25)	ACP (2.45)	Prodrug (2.50)

All doses ( ) are in mmol/kg throughout.

with *N*-AcC, the recognized standard for all cysteine prodrugs.

These two series of L-cysteine double-prodrugs (Charts 2 and 3) were evaluated for their hepatoprotective properties in mice treated with 2.45 mmol/kg of ACP, a dose somewhat lower than that used previously [18] in order to allow for 24 h survival of the animals. Because two sequential steps are necessary for cysteine release by these double-prodrugs, and this was anticipated to span a finite time in vivo, a protocol using divided doses of the hepatoprotective agent administered pre- and post-ACP was implemented (Table 1). Also, since most of the double-prodrugs to be tested had limited aqueous solubility, 2% carboxymethyl cellulose (CMC) was used initially as a suspending agent. However, we found, to our chagrin, that the use of 2% CMC for multiple dose schedules was detrimental (*vide infra*); hence, the prodrugs were dissolved in dilute aqueous base and adjusted to pH 6–8 for administration.

This structure–activity study demonstrated for the first time that there is substantial differential efficacy among the thiazolidine double-prodrugs, as well as among the open-chain *N,S*-diacylated double-prodrugs of L-cysteine, in protecting mice against ACP-induced hepatotoxicity. Indeed, the stringent functional group (pro-moiety) requirement exhibited for maximal efficacy in vivo suggests that differential bioactivation mechanisms must play major roles in the ultimate release of L-cysteine from these double-prodrugs.

MATERIALS AND METHODS

Chemicals

TCA, *N*-AcC (Aldrich, Milwaukee, WI), and OTCA (Chemical Dynamics, South Plaines, NJ) were commercial products used as received, while MTCA, PTCA [5], *N*-AcOTCA, mp 155–158°C, $[\alpha]^{22}_D$ – 139.8° (*c* 1.9, acetone/H_2O, 1:1) reported mp 153–154°C, $[\alpha]^{20}_D$ – 140.5° (*c* 1.8, acetone/H_2O, 1:1 [19]), and *N,S*-bis-acetyl-L-cysteine (*N,S*-bis-AcC), mp 121.5–123.5°C (reported mp 120–121°C) [20], were prepared according to literature methods. The syntheses of the other double-prodrugs of L-cysteine are described later.

Physicochemical/Analytical Methods

^1H NMR spectra were recorded at ambient temperature on Varian Unity 200 and 300 MHz NMR spectrometers equipped with four nuclear probes. Chemical shifts are reported as δ values (ppm). Melting points were taken on a Fisher–Johns hot-stage melting point apparatus and are uncorrected. For TLC analyses, Analtech silica gel GF plates were used. The plates were visualized by spraying with ninhydrin or $CeSO_4$–H_2SO_4 solution and heating. Column chromatography was carried out using columns packed with Kieselgel 60 silica gel (230–400 mesh, EM Science). When the reactants or desired products contained a free sulfhydryl group, the reactions were conducted under a N_2 atmosphere.

Synthetic Procedures

N-Acetyl-2(R,S)-methylthiazolidine-4(R)-carboxylic Acid (N-AcMTCA)

This compound was prepared by acetylation of MTCA (1.47 g, 10.0 mmol) dissolved in 25 mL of 6% aqueous Na_2CO_3 (cooled on an ice bath) by dropwise addition of 2.04 g (20 mmol) of acetic anhydride over 2 min with stirring. After 1 h, the product was isolated by acidification of the reaction mixture, saturating it with NaCl and extracting twice with 50 mL of EtOAc. The combined EtOAc extracts were washed with saturated NaCl, dried (Na_2SO_4), and the solvent evaporated *in vacuo* to give a solid product, mp 164–165°C. Recrystallization from EtOAc gave *N*-AcMTCA as colorless crystals, mp 166–167°C (subl > 150°C) (71% yield). IR (KBr; cm^{-1}) ν = 1730 (CO), 1599 (amide I). The NMR spectrum indicated that the product was approximately a 55:45 mixture of C-2 epimers, which, however, did not separate on TLC, using *n*-BuOH/HOAc/H_2O (100:22:5) (bright yellow on exposure to I_2). ^1H NMR (DMSO) δ 1.42 (d, J = 6.2 Hz) and 1.53 (d, J = 6.4 Hz) (3H, CH_3), 1.98 (s) and 2.09 (s) (3H, CH_3CO), 3.18–3.84 (m, 2H, CH_2), 4.64 (t, 1H, CH), 5.27 (q, J = 6.2 Hz) and 5.35 (q, J = 6.4 Hz) (1H, CH), ^{13}C NMR (DMSO) δ 22.3, 22.9, 23.5, 24.7, 31.9, 33.7, 59.1, 60.2, 62.8, 63.3, 167.8, 168.6, 172.3, 172.6. Anal calcd for $C_7H_{11}NO_3S$: C, 44.43; H, 5.86; N, 7.40. Found: C, 44.60; H, 5.83; N, 7.43.

S-Carbethoxy-N-acetyl-L-cysteine (S-Cbe-N-AcC)

An aqueous solution of *N*-AcC (1.01 g, 6.18 mmol) in an ice bath was stirred under N_2. To this was added

a solution of ethyl chloroformate (0.60 mL, 0.68 g, 6.3 mmol) in ice-cooled THF, followed by 13 mL of an ice-cooled aqueous solution of Na_2CO_3 (663 mg, 6.26 mmol) over 5 min. After ca. 10 min, an additional portion of ethyl chloroformate (0.08 mL) was added for a total of 0.68 mL (0.77 g, 7.1 mmol). The reaction was stirred for an additional 30 min and the solvents were removed *in vacuo*. The product was dissolved in ca. 50 mL of H_2O (pH ~8.0), and the solution was washed with 3 × 20 mL portions of EtOAc and then acidified to pH 3.0 with 1 N HCl. The aqueous solution was extracted with 3 × 20 mL portions of EtOAc. The combined EtOAc extracts were dried and evaporated to dryness *in vacuo* to give a viscous oil. This was lyophilized to give 940 mg (64.7% yield) of *S*-Cbe-*N*-AcC as a viscous, colorless oil which solidified on standing, mp 68.5–71.0°C. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, J = 7.1 Hz, 3H, CH_3CH_2), 2.07 (s, 3H, CH_3CO), 3.29 (dd, J = 6.8, 14.5 Hz) and 3.47 (dd, J = 4.2, 14.5 Hz, 2H, CH_2S), 4.28 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.81 (m, 1H, CH), 6.92 (d, J = 7.3 Hz, NH). ^{13}C NMR ($CDCl_3$) δ 14.3, 22.8, 32.2, 52.9, 64.5, 171.0, 172.1, 172.3. Anal calcd for $C_8H_{13}NO_5S$: C, 40.84; H, 5.57; N, 5.95. Found: C, 40.79; H, 5.47; N, 6.04.

N,S-bis-Carbethoxy-L-cysteine (N,S-bis-CbeC)

L-Cysteine (3.03 g, 25.0 mmol) was stirred under N_2 with cooling (ice bath) in 50 mL of H_2O. Ethyl chloroformate (9.60 mL, 10.9 g, 100 mmol) was added over 2 min, followed by NaOH (4.40 g, 110 mmol) in 50 mL of H_2O over 15 min. The solution was stirred for 30 min (the pH was 8.5), then extracted with 3 × 50 mL portions of EtOAc. The aqueous layer (now pH 7.5) was acidified to pH 2 with 10 mL of 6 N aqueous HCl and extracted with 3 × 50 mL portions of EtOAc. The organic extracts were dried and concentrated to give 7.94 g of viscous oil. The oil was purified by column chromatography, elution being done with EtOAc/hexane (1:1) to give *N,S-bis*-CbeC as a viscous oil (4.87 g, 73.6% yield). This product was subjected to lyophilization to remove traces of residual EtOAc. ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, CH_3CH_2), 3.27 (dd, J = 6.8, 14.4 Hz) and 3.46 (dd, J = 4.6, 14.4 Hz, 2H, CH_2S), 4.13 and 4.28 (q, J = 7.1 Hz, 4H, CH_2CH_3), 4.60 (m, 1H, CH), 5.66 (d, J = 7.8 Hz, NH), 6.59 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 14.2, 14.4, 32.7, 53.8, 61.7, 64.3, 156.4, 170.6, 174.2. Anal calcd for $C_9H_{15}NO_6S$: C, 40.75; H, 5.70; N, 5.28. Found: C, 40.62; H, 5.51; N, 5.26.

S-Acetyl-N-carbethoxy-L-cysteine (S-Ac-N-CbeC)

A solution of *N,N'*-bis-carbethoxy-L-cystine, prepared according to a literature procedure [21] (513 mg, 1.33 mmol), in 50 mL of H_2O was stirred under N_2. To this was added a solution of tris carboxyethyl phosphine HCl [22] (532 mg, 1.86 mmol) in 50 mL of N_2-purged H_2O. After 45 min, analysis of an aliquot showed no starting material by TLC and a spot with positive test for SH. The pH of the solution was raised from 2.8 to 10.5 with ~13 mL of 1 N aq NaOH. Acetic anhydride (0.80 mL, 0.86 g, 8.4 mmol) was added over 1 min, after which time the pH of the solution was 4.6. This was raised to 8.2 with additional 12 mL of 1 N aq NaOH. TLC analysis after ca. 30 min showed that no products with free thiol remained. The solution was acidified to pH 2.7 with 6 N aq HCl and extracted with 3 × 70 mL portions of EtOAc. The combined EtOAc extracts were dried (Na_2SO_4) and concentrated to give 667 mg of oil. This was purified by column chromatography, elution being done with EtOAc/hexane (1:1) to give *S-Ac-N*-CbeC as a colorless oil (334 mg, 53.4% overall yield) which solidified on standing, mp 86–88°C. ^{1}H NMR ($CDCl_3$) δ 1.25 (t, J = 7.1 Hz, 3H, CH_3CH_2), 2.37 (s, 3H, CH_3CO), 3.33 (dd, J = 7.0, 14.3 Hz) and 3.47 (dd, J = 4.5, 14.2 Hz, 2H, CH_2S), 4.14 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.6 (m, 1H, CH), 5.56 (d, J = 7.7 Hz, NH), 9.48 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 14.5, 30.6, 30.9, 53.7, 61.8, 156.5, 174.2, 195.8. Anal calcd for $C_9H_{13}NO_5S$: C, 40.84; H, 5.57; N, 5.95. Found: C, 40.94; H, 5.53; N, 5.96.

N-Carbethoxy-L-cysteine (N-CbeC)

N,N-bis-Carbethoxy-L-cystine (505 mg, 1.31 mmol) in 50 mL of H_2O was reduced as aforementioned with 500 mg (1.74 mmol) of the phosphine reagent, and the reaction solution (pH 2.1) was extracted with 3 × 50 mL portions of N_2-purged EtOAc. The combined EtOAc extracts were dried and concentrated to give 495 mg of a viscous oil. This was purified by chromatography, elution being done with EtOAc/hexane (1:1) and EtOAc to give *N*-CbeC as a colorless oil (262 mg, 52% yield). The column was maintained under N_2 and the chromatography solvents were all flushed with N_2 before use. ^{1}H NMR ($CDCl_3$) δ 1.26 (t, J = 7.1 Hz, 3H, CH_3CH_2), 3.0 (m, 2H, CH_2S), 4.14 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.7 (m, 1H, CH), 5.59 (d, J = 7.8 Hz, NH), 9.53 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 14.5, 27.0, 54.9, 61.8, 156.3, 174.3. Anal calcd for $C_6H_{11}NO_4S$: C, 37.30; H, 5.74; N, 7.25. Found: C, 37.45; H, 5.53; N, 7.14.

Animals

Male, Swiss-Webster N4D mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 25–34 g were housed and cared for in conventional cages according to the guidelines of our Institutional Animal Care and Use Committee (IACUC), with a 12:12 light/dark

photoperiod (lights on at 0700) in a temperature controlled (21–22°C) room. Water and food (Harlan Rodent Chow) were allowed *ad libitum* except as noted later. All of the following protocols were IACUC approved.

Drug Administration Protocols

Following an acclimatization period of 5–12 days, the mice were fasted overnight (16 h) before drug administration by intraperitoneal (i.p.) injection. The details of the various drug dose protocols are listed in Table 1. In order to manage the large numbers of prodrugs to be tested and to minimize any fluctuations in animal responses on a given day, the animals were divided into groups of 3 or 4 for each protocol, and the experiment repeated several days later to reach the n required for statistical treatment. When excessive toxicity was evident—as manifested by unexpected premature deaths—the experiments with those prodrugs were terminated as required by our IACUC (Protocols 1 and 2).

The prodrugs were prepared as a suspension in either 2% carboxymethyl cellulose (CMC) or as a solution in a pH adjusted aqueous medium (pH 6–8) as follows. Crystalline samples of the prodrugs were weighed and ground to a fine powder in a quartz mortar. (This initial grinding was not necessary for prodrugs that were oils or gums.) To this was added 0.25 mL of 2% CMC and a slurry was produced by grinding to ensure a fine suspension. An additional 0.25 mL of suspending agent was added, followed by further grinding. The suspension or slurry was brought to the final volume with 2% CMC.

All of the prodrugs tested in this series could be solubilized in dilute aqueous base. Accordingly, depending on the alkaline stability of the compound, 0.25 mL of either 10 N or 1 N NaOH was added to the finely ground, preweighed sample. A slurry was produced by grinding with a pestle and another 0.1 mL of base was added to increase the volume, followed by incremental additions of base until clear. The solution, usually 0.4–0.5 mL, was transferred into a microfuge tube (1.5 mL) and the pH determined using a microprobe. Additional base was added to adjust pH, and when necessary, dilute HCl to back titrate to pH 6–8. The solution was then brought to volume with saline to give the final stock solution for injections.

ACP, dissolved in warm saline, was administered at zero time at 0.015 mL/g body weight, which corresponded to a dose of 360 mg (2.45 mmol)/kg. We found it necessary to reduce the dose of ACP from the 400 mg (2.65 mmol)/kg dose used previously [18] because these mice (although of the same strain purchased from the same source) appeared to be much more sensitive to ACP toxicity. Even this lower dose appeared to be toxic, except for those mice fully protected by the most effective prodrugs (*vide infra*). The animals were allowed access to food following the last drug administration, observed for 6 h post-ACP, and, when deemed necessary, were sacrificed on imminent death, or blood was drawn shortly following death, for measurement of alanine aminotransferase (ALT) levels (*vide infra*). ALT levels were not determined for mice that died between 6–24 h; however, the numbers of such deaths within a group were recorded.

Plasma ALT Levels

Twenty-four hours following the administration of ACP, the mice were anesthetized with Ketamine/Xylazine (3:1), and blood was collected by cardiac puncture. Samples were placed in heparinized Wintrobe tubes, centrifuged in a Sorvall RC-3B refrigerated centrifuge for 10 min (H2000 rotor, 1500 rpm, 5°C), and the plasma layer collected and kept refrigerated until ALT activities (U/L) were determined (in duplicate) by kinetic assay (30°C), using Sigma Infinity ALT Reagent and a Beckman Model DU-70 spectrophotometer. Plasma samples that required storage longer than overnight were kept in an ultra-low freezer at −80°C. The ALT activities of samples stored at this temperature have been reported to be stable for at least a week [23].

Data Analysis

The ACP dose selected for this animal model (2.45 mmol/kg), although reduced from previous studies (see Drug Administration Protocols), induced high plasma ALT levels within 24 h. To determine whether the prodrugs protected against ACP-induced hepatotoxicity, the averaged plasma ALT values from mice from each prodrug group were log transformed [24] and the 95% and 99% confidence intervals of the log values were calculated, then plotted for each group and compared to the ACP group and the vehicle control group [18]. The differences between groups (at the corresponding p value) were considered significant when their confidence intervals did *not* overlap.

RESULTS

The hepatoprotective properties of the thiazolidine double-prodrugs of L-cysteine administered pre- and post-ACP in divided doses (Protocol 1, Table 1), as reflected by plasma ALT levels measured 24 h following the administration of ACP, are shown in Figure 1. Their relative degrees of efficacy are readily compared by

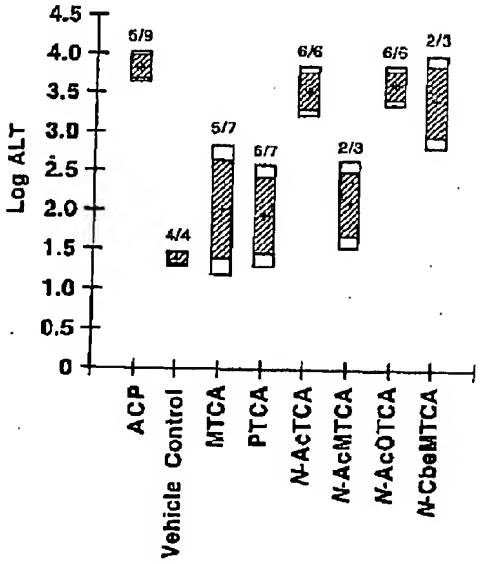


FIGURE 1. Protection from ACP-induced hepatotoxicity by thiazolidine prodrugs and double-prodrugs of L-cysteine, using CMC as vehicle (Protocol 1, Table 1). Survival rates (24 h) are as indicated. See Materials and Methods section for details on how the data were analyzed.

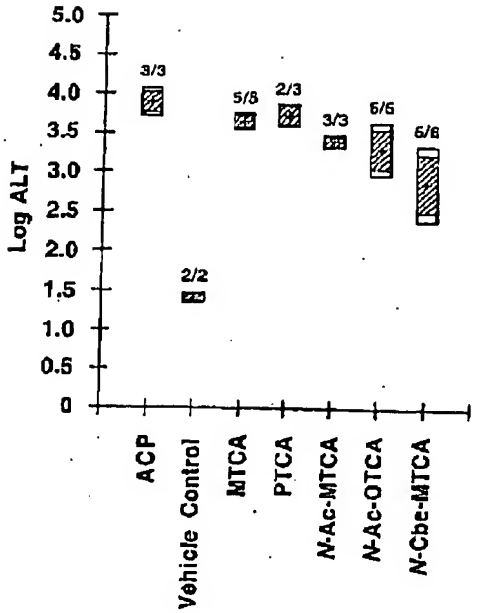


FIGURE 2. Lack of hepatoprotection by thiazolidine prodrugs and double-prodrugs of L-cysteine when preadministered in CMC (Protocol 2, Table 1). Also see legend to Figure 1.

visual inspection of the overlap of the 95% (unhatched) and/or 99% (hatched) confidence intervals of the log transformed data [18] (use of a straight edge is suggested). The rationale for the use of this paradigm for screening of large numbers of potential hepatoprotective agents in mice was to keep the number of animals required at minimal levels, yet be within the acceptable parameters for valid statistical treatment of the data.

The lack of protection by *N*-AcTCA, *N*-AcOTCA, and *N*-Cbe-MTCA can be seen readily. Notable, however, was the apparent poor efficacy displayed by the positive controls, MTCA and PTCA, compounds known to be highly effective in protecting mice against ACP-induced hepatotoxicity [4,25] as well as ACP-[26] and naphthalene-induced cataractogenesis [27]. This raised the question whether this pretreatment protocol, especially the use of CMC as vehicle for repeated injections, was problematical. This suspicion was verified when the results of experiments using Protocol 2 (Table 1) were analyzed. In this protocol, only the pretreatment schedule with the prodrug was retained and the second, post-treatment dose following ACP was eliminated. The data of Figure 2 clearly indicate that a *single pretreatment schedule* with prodrug suspended in CMC and administered 1 h prior to ACP, *even at a dose which was highly effective as a single dose given 30 min post-ACP* [18], was totally ineffective in protecting mice against hepatotoxicity elicited by ACP.

The use of CMC as a dispersing agent now contraindicated, it was necessary to employ another dispersing agent or to somehow solubilize these cysteine double-prodrugs in order to facilitate their administration. Fortunately, a uniform characteristic of all these prodrugs was the presence of a free carboxyl group in the molecule with potential for salt formation. Accordingly, stock injection solutions of the prodrug were prepared by dissolving them in dilute NaOH and then adjusting the pH to near neutrality (see Materials and Methods section). The results of protection experiments using this Protocol 3 (Table 1) are presented in Figure 3. *N*-AcC was included here as the positive control for the open-chain cysteine prodrugs. The results indicated that except for *N,S*-bis-AcC none of the double-prodrugs of cysteine, either as thiazolidine forms or as open-chain derivatives, was effective in protecting mice against ACP-induced hepatotoxicity.

While these results were disappointing, most troubling was the observation that the three positive controls, viz., MTCA, PTCA, and *N*-AcC, all displayed less than optimal protective activity with this protocol. It was now clear from the results of Figure 3 that the pre- and post-ACP doses of prodrugs, viz., the full dose and half dose, respectively, of Protocol 3 (Table 1),

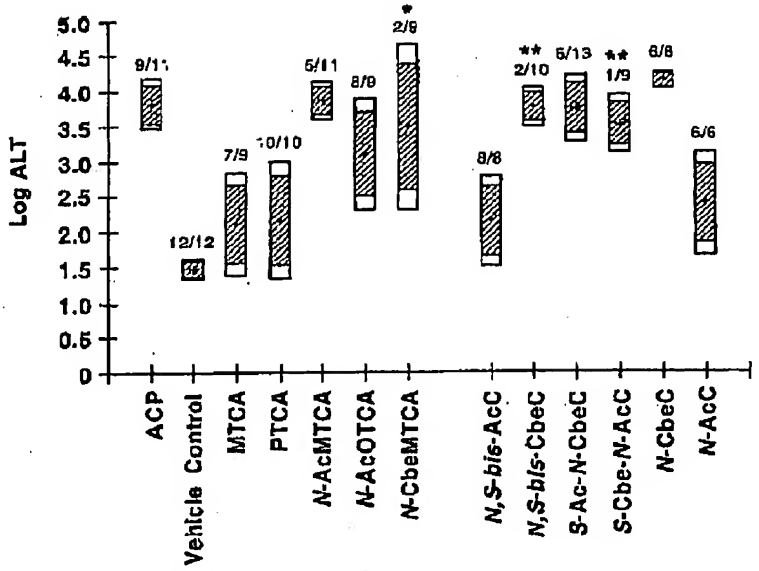


FIGURE 3. Protective effects of a series of prodrugs and double-prodrugs of L-cysteine against ACP-induced hepatotoxicity, using Protocol 3 (Table 1). Also see legend to Figure 1. *: Blood from 5 animals only. **: Blood was available from 7 animals.

needed to be *reversed* for maximal efficacy, and Protocol 4 was established as the ultimate paradigm for implementation. The results of this study are graphically summarized in Figure 4. Significantly, the ALT levels of ACP-mice treated also with MTCA and PTCA, the positive controls for the thiazolidine double-prodrugs, were now *not different* from the ALT levels of the vehicle control animals at the 99% and 95% confidence levels, respectively, while the ALT levels of the N-AcC-treated animals, the positive control for the open-chain

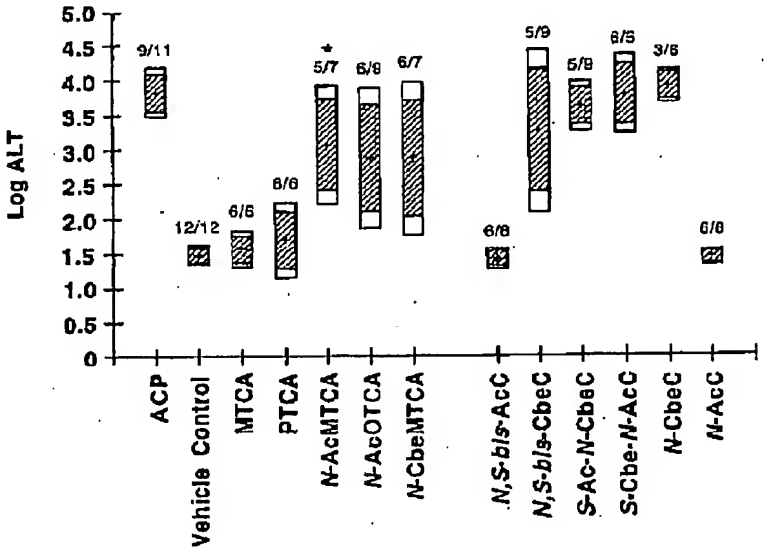


FIGURE 4. Differential protection against ACP-induced hepatotoxicity by double-prodrugs of L-cysteine (Protocol 4, Table 1). Also see legend to Figure 1. *: Blood was available from 6 animals.

double-prodrugs of cysteine, were not different from vehicle control mice at the 99% confidence level.

The hepatoprotection displayed by the *N*-acylated thiazolidine double-prodrugs of L-cysteine was marginal at best, (Figure 4), and while several of the mice in each group were fully protected based on plasma ALT levels at 24 h, premature deaths were also recorded. Thus, the *N*-acyl group, in particular, the *N*-acetyl group (*vide infra* for discussion of the *N*-Cbe group) on the thiazolidines appears to be metabolically much more stable than had been anticipated.

The standout among all of the double-prodrugs tested was *N,S*-bis-AcC, which fully protected mice from hepatotoxicity (Figure 4). In marked contrast, *N,S*-bis-CbeC and *N*-CbeC itself, where the *acetyl* groups were replaced with the *carbethoxy* group, were totally ineffective in protecting mice against ACP-induced hepatotoxicity. While no deaths were encountered with MTCA, PTCA, *N,S*-bis-AcC, or *N*-AcC, which were all fully protective, many of the animals treated with *N,S*-bis-CbeC and/or with *N*-CbeC died prematurely (before 6 h) requiring that blood be drawn soon after death for the measurement of plasma ALT levels (Figure 4).

DISCUSSION

In previous studies, MTCA, suspended in 2% CMC and administered as a *single* dose 30 min *post*-ACP, protected against hepatotoxicity [18]. However, in the present study, the levels of protection achieved by MTCA and PTCA also suspended in 2% CMC but administered pre-and post-ACP (Protocol 1, Table 1) were highly erratic (Figure 1). This lack of effective protection observed even with MTCA and PTCA appears to be due to the detrimental effects of CMC itself. For example, it has been reported that viscous additives such as CMC used as carrier can adversely affect drug absorption [28]. Moreover, pretreatment of mice with 1% CMC before challenge with ACP (300 mg/kg) was shown to reduce liver GSH concentration by 81% and dramatically increase liver injury [29]. Our results using Protocols 1 and 2 (Figures 1 and 2) could, therefore, be consequences of this CMC effect coupled to the inherent hepatotoxicity of ACP. Thus, as documented here, the use of CMC as a dispersing agent in the evaluation of hepatoprotective agents is contraindicated, despite previous successes with its use.

Roquebert et al. [30] reported that when the pyridoxine salt of *N*-acetyl-TCA (*N*-AcTCA, structure not shown) radiolabeled with ^{35}S was administered to rats, *N*-AcTCA was recovered unchanged in the urine. We have observed that a large dose of *N*-AcMTCA administered i.p. to rats (1.25 g/kg) can also be recovered unchanged in the urine (unpublished). Although the urinary recoveries were not quantitative, these data suggest that the acetyl groups on *N*-AcTCA and *N*-AcMTCA are not readily hydrolyzed in vivo, reflecting the observed lack of hepatoprotection by *N*-AcTCA (Figure 1) and the marginal activity of *N*-AcMTCA (Figure 4). On the other hand, the acetyl group in *N*-AcOTCA, being part of an imide structure, should have been enzymatically more labile, the expected product here being OTCA, a known hepatoprotective cysteine prodrug [3]. The large variation in efficacy displayed by this compound (Figure 4) suggests that individual animals must have shown considerable differences in the double enzymatic deacetylation and thiazolidine ring opening steps leading to the release of L-cysteine.

Conceptually, double-prodrugs should confer high selectivity to the drugs being latentiated, since two sequential enzymatic steps are required for their ultimate liberation in vivo, and specificity for each step should vary, depending on the enzymatic makeup of the species, strain, etc. The use of random-bred Swiss-Webster mice instead of an inbred mouse strain to evaluate the efficacy of this series of cysteine double-prodrugs was a deliberate effort to assess the universality of action of these compounds. Double-prodrugs that exhibit high hepatoprotective properties in this outbred mouse strain would likely be more reflective of and applicable to the human situation, the goal strived for in drug research. Conversely, double-prodrugs with poor efficacy due to differential specificity at each step, a consequence of genetic polymorphism [31], should be avoided for further development.

A case in point is the carbethoxy (Cbe) group. This group, whether attached to divalent sulfur (as in *S*-Cbe-*N*-AcC) or on nitrogen (as in *N*-CbeC or *N*-Cbe-MTCA) can, theoretically be *hydrolyzed* by esterases or be *oxidatively removed* by the cytochrome P450 enzymes via ethyl group dealkylation, both routes giving rise to CO_2, as well as ethanol and acetaldehyde, respectively, as by-products. The fact that those compounds functionalized with the Cbe group had erratic, widely differential hepatoprotective efficacy (Figure 4) suggests that polymorphism must play a major role in the liberation of cysteine from these Cbe-functionalized double-prodrugs. In marked contrast, *N,S*-bis-AcC, where both the sulfhydryl and amino groups of L-cysteine are functionalized with the acetyl group, was highly effective in protecting mice from the hepatotoxic effect of ACP (Figure 4). It follows that enzymatic removal of the acetyl groups from *N,S*-bis-AcC was facile and was not subject to differential metabolism because of genetic polymorphism.

Whether the activity of these cysteine prodrugs is limited to their stimulatory action on the biosynthesis of GSH and its sequestration of NAPQI produced in the oxidative metabolism of ACP remains unclear. The

nullification by GSH of the various reactive oxygen and nitrogen species, which are the toxic mediators in the early stages of the inflammatory cascade, may also play a role. It is known that maintaining GSH homeostasis protects the cell from depletion of AdoMet by protecting the enzyme, AdoMet synthetase (methionine adenosyl transferase), required for its biosynthesis [32]. AdoMet synthetase, being a sulfhydryl enzyme, requires GSH homeostasis to maintain the integrity of its active-site sulfhydryl groups [33]. It is also a key enzyme in the methylation cycle with major cellular functions [34]. We have recently shown that ACP administration to mice not only depletes hepatic mitochondrial GSH levels, but also compromises the activity of AdoMet synthetase; however, the administration of L-cysteine prodrugs protected the activity of this enzyme [35]. Carbon tetrachloride [32] and *N*-ethylmaleimide [36] are also known to adversely affect the activity of AdoMet synthetase. It is of interest that AdoMet administration to mice protects against ACP-induced hepatotoxicity [37,38].

The physiological and biochemical responses to cellular injury induced by chemical agents such as ACP are complex, and the effective protection provided by an agent that ensures survival most likely attenuates responses to oxidative stress in other organ systems, besides the liver. Thus, for effective treatment, the lethal consequences of macrophage hyperactivation that are manifested early in the induction of toxicity must also be counteracted [39–41]. Whether postadministration, e.g., 4–12 h *after* ACP, of these prodrug forms of GSH can attenuate the cellular effects of cytokine activation has never been addressed, but such experiments are being seriously contemplated, since recent studies have shown that OTCA treatment attenuated the production of the proinflammatory cytokines TNF-α and IL-2 in rats chronically administered ethanol intragastrically [42].

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